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PRINCIPAL INVESTIGATOR: Matthew R. Swift

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Washington, DC 20007

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Matthew R. Swift

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: Mrs8@georgetown.edu

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13. ABSTRACT (Maximum 200 Words)

Fibroblast growth factor binding protein (FGF-BP1) is a crucial molecule that acts to chaperone active FGFs to receptors, thus propagating angiogenic signals for the development of new vasculature. We have shown that FGF-BP1 is expressed in head and neck, skin, cervical, and lung squamous cell carcinomas. A second family member, FGF-BP2 has been identified in our lab and is present in mammary tissue. In this grant, we hypothesized that FGF-BP2 acts in a similar pro-angiogenic capacity as FGF-BP1. The aims were 1) to produce recombinant FGF-BP2 and test its effect on signal transduction, and 2) to study the expression of FGF-BP2 during mouse mammary gland development and carcinogenesis. To date, we have isolated human FGF-BP2 cDNA and protein and confirmed its ability to modulate FGF2. However, we have yet to discover the murine homologue to FGF-BP2. We have identified a third family member, FGF-BP3 in human and mouse. Accordingly, we have adjusted our focus to the characterization of FGF-BP3 activity and expression while continuing our search for murine FGF-BP2. Both human and murine FGF-BP3 have been shown to bind FGF2, promote increased proliferation, MAPK activation, and anchorage-independent growth in SW-13 adrenal carcinoma cells, and murine FGF-BP3 is present in high levels at specific time points in developing mouse embryonic tissue.

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Introductions

Tumor growth is dependent upon local-acting growth factors to stimulate the infiltration and growth of new blood vessels from surrounding normal tissue into the tumor mass, a process known as angiogenesis [1,2]. An important class of growth factors that positively regulate angiogenesis is the fibroblast growth factor (FGF) family [3]. Two key members of this family, FGF-1 and FGF-2, require a chaperone molecule to release these factors from extracellular storage and present them to target receptors. These chaperone molecules are known as FGF-binding proteins (FGF-BPs). Two members of the FGF-BP family have been identified so far, FGF-BP1 and FGF-BP2 [4,5]. Previous work from our laboratory has shown that FGF-BP1 can positively modulate the biological activity of FGFs, can support tumor growth and angiogenesis in FGF-2 expressing cell lines, is highly expressed in multiple cancers, and can act as an angiogenic switch *in vivo* in colon cancer cells and SCC. [6,7]. Preliminary data from our lab suggests that FGF-BP2 may act in a similar fashion to chaperone FGFs but has shown distinct expression patterns in tumor samples, particular in human breast cancer samples. A crucial aspect to our study was examination of the expression of FGF-BP2 during mouse development and carcinogenesis, but to date, no murine homologue for FGF-BP2 has been found. Interestingly, a third FGF-BP family member, FGF-BP3 has recently been identified in our lab and a murine homologue for FGF-BP3 was also found. In this study, we began to examine the biological effects of FGF-BP2 but having found mouse and human FGF-BP3, we have refocused our study to explore the effects of this new family member.

Body

We have previously discovered and investigated a new member of the FGF-BP family, FGF-BP2. In an effort to study the biological significance of human FGF-BP2, our goals outlined in Aim 2 were contingent upon the isolation of a murine homologue to FGF-BP2. However, one candidate murine EST thought to contain FGF-BP2 that was isolated through homology searches using the human and chicken FGF-BP2 gene proved instead to be the murine homologue of just discovered human FGF-BP3 (**Fig 1a**). Accordingly, our research aims have been adjusted to investigate the activity of human and murine FGF-BP3 through many of the processes previously described for FGF-BP2. Although we have progressed with the examination of FGF-BP3, our efforts to identify and isolate a murine homologue to FGF-BP2 have continued, but yielded no discoveries. During the course of our search, we have identified a previously unknown chicken homologue to FGF-BP1 providing us with an updated radial tree depicting the phylogenic relationship of all known members of the FGF-BP family (**Fig 1b**).

The goals stated in Aim 1 have now been modified to reflect the initial characterization of FGF-BP3. Both human and murine FGF-BP3 have been isolated, amplified, and expressed in a pcDNA3.1 vector construct containing both a V5 and poly-histidine carboxy-terminal tag. Please note that no attempts at production of a recombinant FGF-BP3 protein will be made in this project. As reported previously, SW-13 cells engineered to express human FGF-BP3 exhibited an increased level of proliferation with a concurrent increase in MAPK phosphorylation and showed increased levels of anchorage-independent growth over control as determined through colony formation. A neutralizing antibody towards FGF2 eliminated the increased colony formation showing this effect to be FGF2-dependent (**Fig 2**). Preliminary studies with

newly engineered SW-13 cells now expressing murine FGF-BP3 show similar results in both proliferation and anchorage independent growth. A more detailed investigation with proliferation and soft agar assays will be performed to further characterize the biological response of increased human and murine FGF-BP3 expression upon a variety of pro and anti-mitogenic stimuli. Previously, immunoprecipitation assays suggested specific complex formation between human FGF-BP3 and FGF2. Further attempts to demonstrate this interaction were unsuccessful. Accordingly, a cell-free binding assay was specifically developed for this project to determine the ability of FGF-BP3 to bind FGFs. Early results show that concentrated conditioned media containing either human or murine FGF-BP3 is able to specifically bind immobilized FGF2 (**Fig 3**). With this protocol now established, we can seek out a more complete binding profile for the ability of FGF-BP3 to bind additional FGFs as well as other growth factors. Also, competition assays can be employed to help establish the affinity of FGF-BP3 for these various growth factors. In keeping with the initial goals of Aim 1, we will also continue to investigate the role of FGF-BP3 in signal transduction pathways, specifically those that are pro-angiogenic. **Our data indicates that both human and murine FGF-BP3 can mimic the ability of FGF-BP1 to bind FGF2 and can enhance FGF2-dependent biological activity in SW-13 cells.**

The goals of Aim 2 have essentially remained intact but now focus on the expression of FGF-BP3 mRNA during total mouse development. Previous experiments with appropriate controls indicated a ubiquitous rather than specific FGF-BP3 mRNA staining pattern in 11-day old mouse embryo sections. In an attempt to isolate a better time-point to study FGF-BP3 mRNA expression in developing mouse embryos, Northern Blot analysis was undertaken on tissue isolated from various stages of development.

Interestingly, our analysis shows a remarkable spike of FGF-BP3 expression at day 10.5 with a subsequent drop-off in expression around day 14.5 (**Fig 4**). Furthermore, *in situ* hybridization analysis in mouse embryo sections from day 8 though 16 reinforce this finding with earliest staining observed at day 9 and a near complete lack of staining by day 14. Currently, we are having these slides analyzed to determine specific regions of FGF-BP3 staining in the earlier time points prior to the ubiquitous staining pattern observed by day 10-11. Aim II, Series #2 has also been adjusted to allow for the creation of peptide antibodies against regions of both human and mouse FGF-BP3. Specifically, one set of antibodies have been targeted to a 12-mer found only in human FGF-BP3, named "HUM BP3", while a second set of antibodies have been targeted to another 12-mer with 100% homology between both human and mouse FGF-BP3, "PAN BP3" (**Fig 1a**). Additionally, peptide antibodies have been created against a 12-mer located on the carboxy-terminal end of human FGF-BP2 that will help with our continuing search for its biological activity. As we await the full development of all antibodies, early unpurified 8-week bleeds from selected rabbits with the HUM BP3 antibody have shown it to be able to specifically identify human and not mouse FGF-BP3 in the conditioned media of transfected SW-13 cells via Immunoblot (**Fig 5**). Further experimentation with human and murine cells lines thought to contain FGF-BP3 are in development as is the expansion of the HUM BP3 peptide antibody for use in immunoprecipitation assays as well as immunohistochemistry analysis. **Our data indicates that FGF-BP3 expression is highly up-regulated during a specific time frame in embryonic development as witnessed through both Northern Blot and *in situ* hybridization analysis.**

A.

```
mBP3        MANAGMSPPRPRASLSPLTLLLLLGGCLLSAAGRDKGAAGREVTRASRPTVGSSGRFVSP 60
hBP3        -----MTPPKLRASLSP-SLLLLLSGCLLAAARREKGAASNVAEPVPGPTGGSSGRFLSP 54
                 *:**: ****** :*****.****:** *:****.. .  .. ** ******:**

mBP3        EQHACSWQLLVPAPGTPTGGELALRCQTPGGASLHCAYRGHPERCAATGARRAHYWRRLL 120
hBP3        EQHACSWQLLLPAPEAAAGSELALRCQSPDGARHQCAYRGHPERCAAYAARRAHFWKQVL 114
            **********:*** :.:*.*******:*.**  :************ .*****:*:::*

mBP3        GALRRRPRPCLDPAPLPPRLCA-RKTAGSDLHSPAHPSLPARPSEP-------PRSRARS 172
hBP3        GGLRKKRRPCHDPAPLQARLCAGKKGHGAELRLVPRASPPARPTVAGFAGESKPRARNRG 174
            *.**:: *** ***** .**** :*  *::*:  .:.* ****:.       **:* *.

mBP3        PARSRQS---VRSP---SSQPEKKPLLVKSNSGGRKAGSDPVPEPPAAAGFQPNGLDQNA 226
hBP3        RTRERASGPAAGTPPPQSAPPKENPSERKTNEGKRKAALVPNEERPMGTGPDPDGLDGNA 234
             :*.* *   . :*   *: *:::*   *:*.* ***.  *  * * .:* :*:*** **

mBP3        ELTETYCTEKWHSLCNFFVNFWNG 250
hBP3        ELTETYCAEKWHSLCNFFVNFWNG 258
            ******:*:****************
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"HUM BP3"

"PAN BP3"

B.

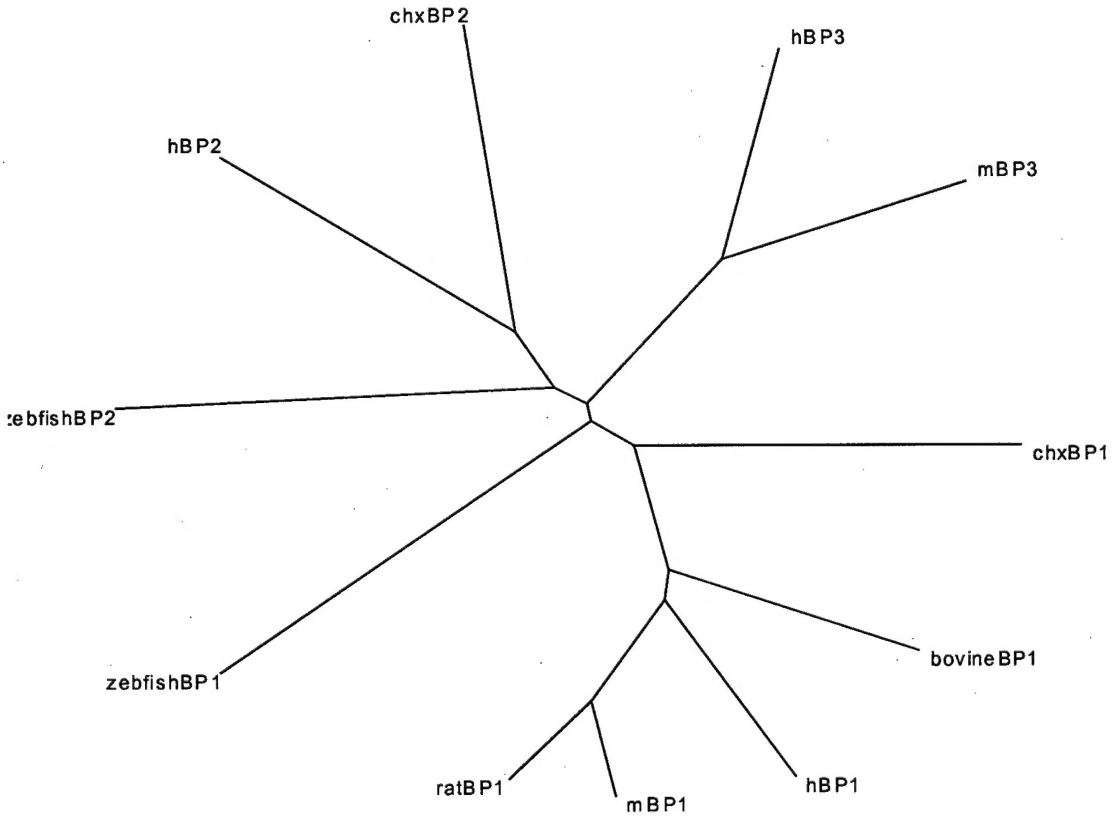

Figure 1: **Phylogenetic relationships of FGF-BP3.** A.) Alignment of the amino acid sequences for human and mouse FGF-BP3. Regions targeted for peptide antibody production are indicated. B.) Updated radial tree diagram demonstrating species homology of the three known members of the FGF-BP family including the recently identified chicken FGF-BP1

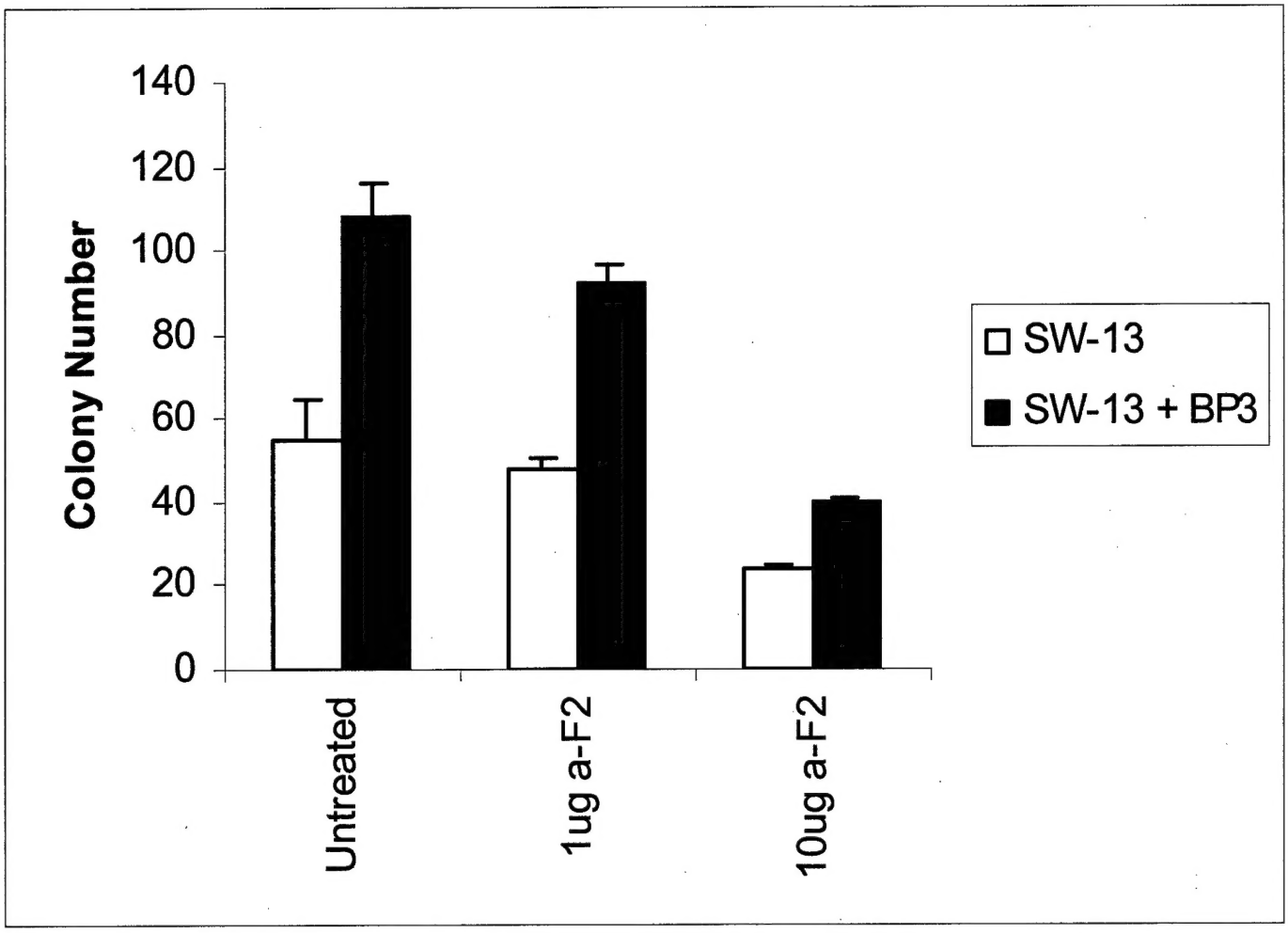

Figure 2: **Human FGF-BP3 can modulate FGF2-dependant growth.** Soft agar assay measuring anchorage-independent growth in parental SW-13 and FGF-BP3-expressing SW-13 cells grown in both the absence and presence of two doses of a neutralizing antibody to FGF-2. Colonies more than 80 μm in diameter were counted after 14 days of incubation

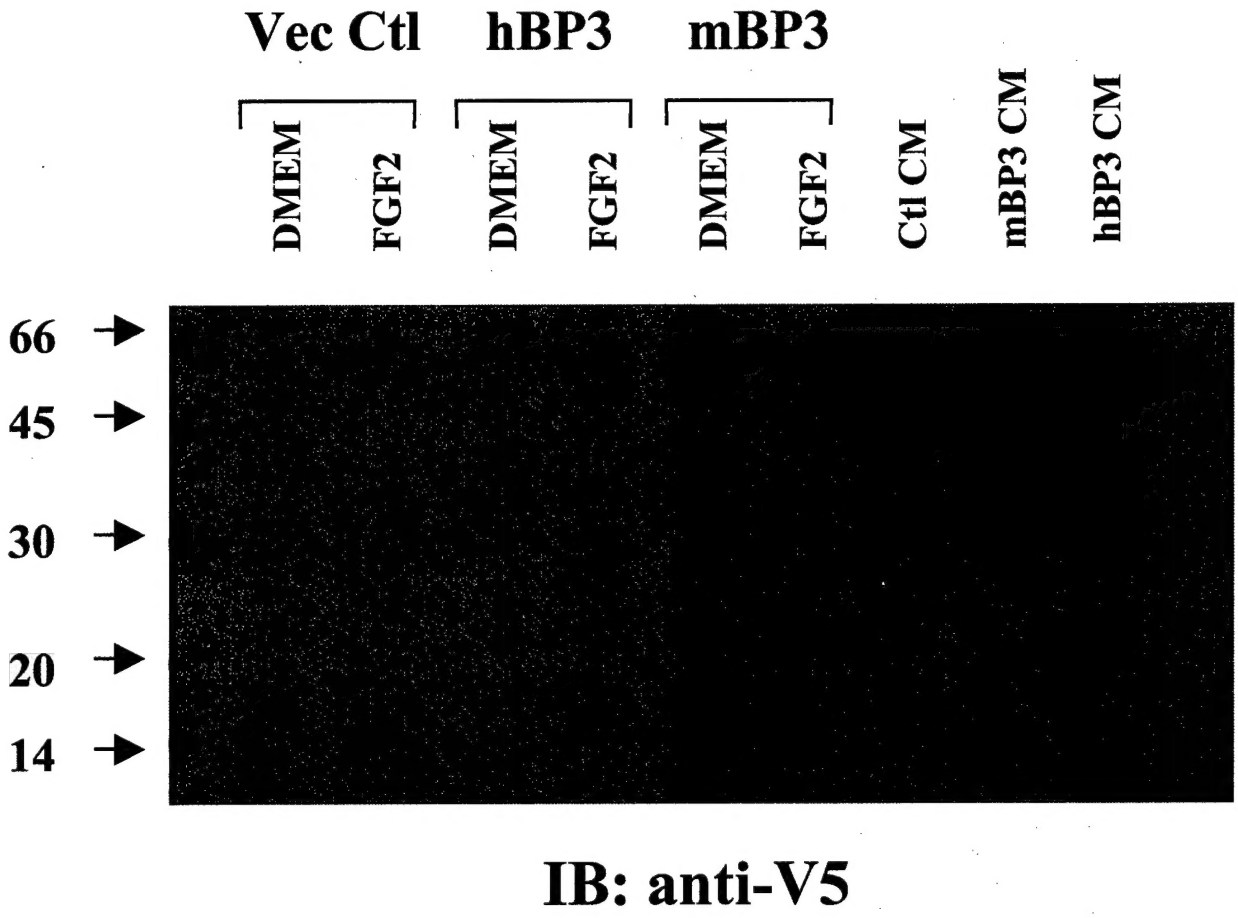

Figure 3: **Cell Free Binding Assay for FGF-BP3 and FGF2 interaction**. Human recombinant FGF2 was immobilized on a 96-well plate overnight. After blocking, concentrated conditioned media with human and mouse FGF-BP3 or control were added for 2 hours. Circles indicate appropriate protein size for both human and mouse FGF-BP3 in wells that contained FGF2 only. Left lanes show approximate amount of FGF-BP3 added.

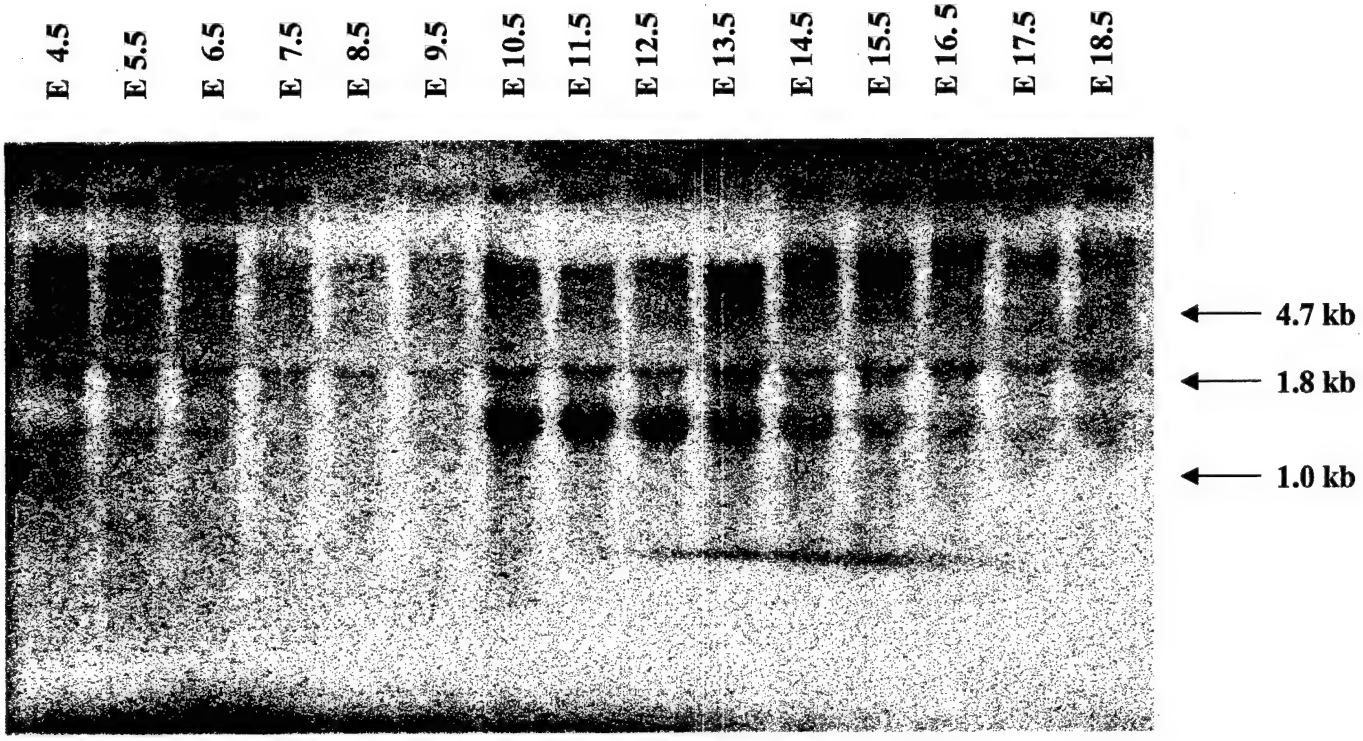

Figure 4: **Northern Blot of Mouse Embryo Tissue Samples**. A pre-made membrane with samples collected from mouse embryonic tissue from day 4.5 to day 18.5 was probed with a specific mouse FGF-BP3 sequence. As indicated at approximate size of 1.2 kb, a dramatic spike in FGF-BP3 expression was observed at day 10.5.

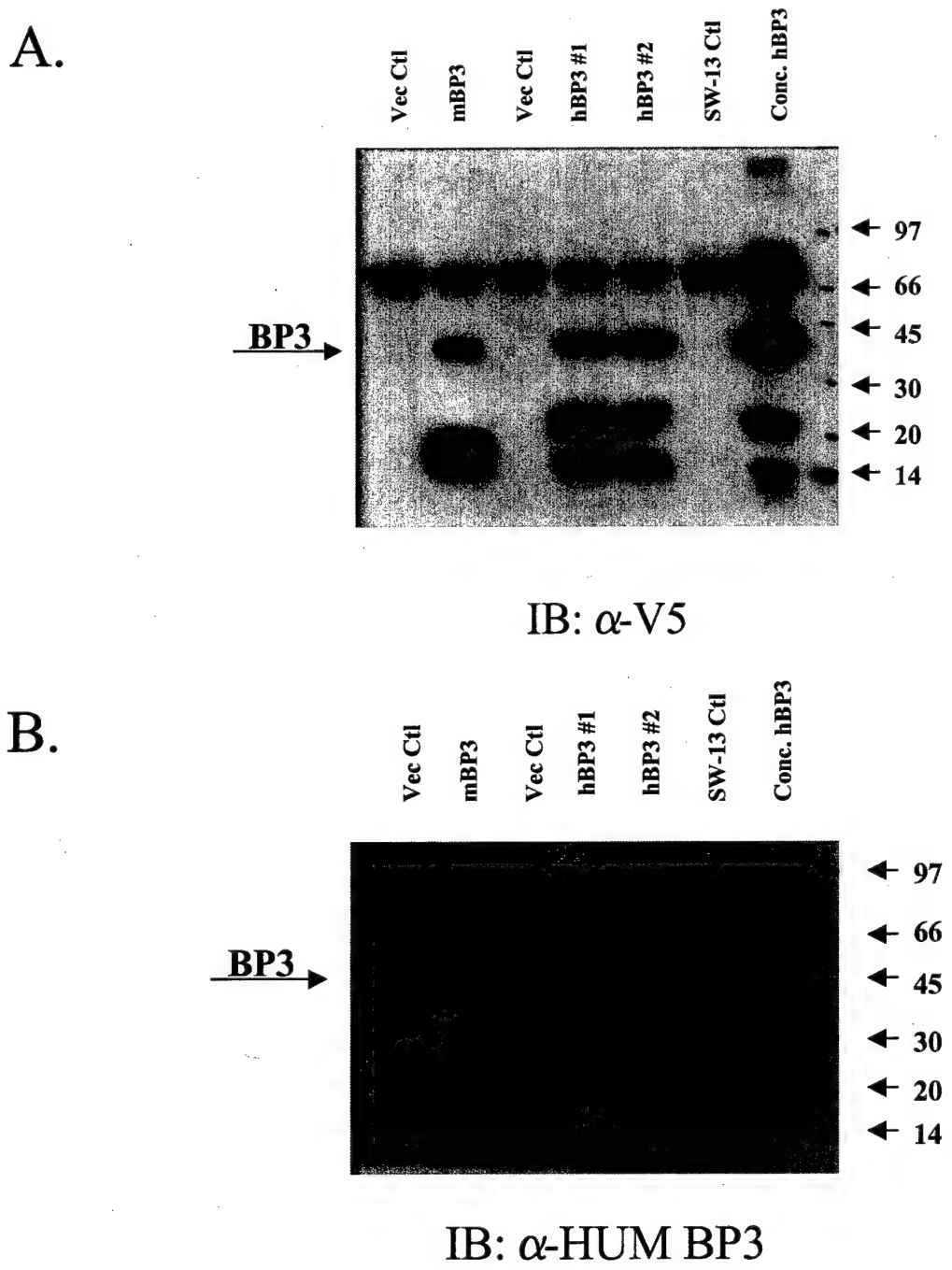

Figure 5: **Immunoblot with a peptide antibody specific for human FGF-BP3.** A.) Conditioned media from SW-13 cells engineered to express human (hBP3) and mouse (mBP3) FGF-BP3, or the vector backbone (Vec Ctl) were subject to Immunoblot analysis against an antibody for the V5 tag. SW-13 control conditioned media (Ctl) and concentrated human FGF-BP3 conditioned media (Conc. hBP3) were used as a negative and positive control respectively. The V5 antibody was able to detect both mouse and human FGF-BP3 constructs. B.) The same samples as above were tested against a peptide antibody specific for human FGF-BP3. The two human FGF-BP3 constructs were detected, however the mouse FGF-BP3 construct was no longer visualized by this peptide antibody.

Key Research Accomplishments

- Identification of a novel Fibroblast Growth Factor Binding Protein, FGF-BP3
- We have isolated and amplified human and mouse FGF-BP3.
- We have generated vector constructs containing the open reading frame of both human and mouse FGF-BP3 and created stable cells lines. These SW-13 cells lines exhibit increased proliferation, MAPK phosphorylation, and anchorage-independent growth over control SW-13 cells.
- We have demonstrated through cell free binding assays that human and mouse FGF-BP3 bind recombinant FGF2.
- We have observed through Northern Blot analysis a significant increase in mouse mRNA expression in embryonic tissue at day 10.5 with a subsequent decrease in mRNA levels through day 16.5.
- We have generated an in situ hybridization probe for human and murine FGF-BP3 and have confirmed the increase in mouse mRNA expression in embryonic tissue slides beginning at day 10 and observed through day 14.

Conclusions

The goal of our initial project was contingent upon the discovery of a murine homologue to human FGF-BP2. The mouse EST sequence we thought to include FGF-BP2 was determined to instead contain FGF-BP3. Due to the recent discoveries of both human and murine FGF-BP3, we have shifted our goal to the characterization of this family member while we continue to search for the murine FGF-BP2. Using stable transfected SW-13 cell lines, we have determined that overexpression of both human and murine FGF-BP3 results in an increased level of proliferation, MAPK phosphorylation, and anchorage-independent growth similar to what has been observed with FGF-BP1 and FGF-BP2 in these same cell lines. As suspected, we have also determined that in cell-free binding assays, human and murine FGF-BP3 is bound by FGF2. Northern Blot analysis with a specific murine FGF-BP3 probe indicates a sizeable increase in mRNA levels in mouse embryonic tissue samples beginning at day 10.5 and diminish through day 16.5. This coincides with ubiquitous staining pattern for FGF-BP3 mRNA was observed via *in situ* hybridization on a mouse embryo slides from day 11 reported previously. Our data was further justified by *in situ* analysis of mouse embryonic slides that ranged from day 8 through day 16, where a sharp increase in mRNA staining began around day 10 and decreased around day 14. We have also initiated the production of peptide antibodies for specific regions of human and mouse FGF-BP3 as well as FGF-BP2. Early results from pre-terminal bleeds have identified an antibody specific for human FGF-BP3 (but not murine FGF-BP3) as seen via Immunoblot analysis. These antibodies, upon completion, should allow us to further investigate biological prevalence and activity of both human and murine FGF-BP3 using Immunoblot, Immunoprecipitation, Immunohistochemistry, and additional analysis.

Abbreviations

FGF: fibroblast growth factor
FGF-BP: fibroblast growth factor binding protein
mBP3: mouse FGF-BP3
hBP3: human FGF-BP3
SCC: squamous cell carcinoma
EST: expressed sequence tag
MAPK: mitogen activated protein kinase

References

1. Folkman J. & Klagsbrun M. (1987) Angiogenic factors. *Science*, **235**, 442.
2. Folkman J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Res*, **46**, 467.
3. Baird A. & Klagsbrun M. (1991) The fibroblast growth factor family. *Cancer Cells*, **3**, 239.
4. Wu D.Q., Kan M.K., Sato G.H., Okamoto T. & Sato J.D. (1991) Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. *J Biol Chem*, **266**, 16778.
5. Powers C.J., McLeskey S.W. & Wellstein A. (2000) Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer*, **7**, 165.
6. Czubayko F., Smith R.V., Chung H.C. & Wellstein A. (1994) Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors. *J Biol Chem*, **269**, 28243.
7. Czubayko F., Liaudet-Coopman E.D., Aigner A., Tuveson A.T., Berchem G.J. & Wellstein A. (1997) A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. *Nat Med*, **3**, 1137.